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Integrating conventional microscopy and molecular analysis to analyse the abundance and distribution of four *Calanus* congeners in the North Atlantic

P. K. LINDEQUE[1]*, S. J. HAY[2], M. R. HEATH[2], A. INGVARSDOTTIR[3], J. RASMUSSEN[2], G. R. SMERDON[1] AND J. J. WANIEK[4]

[1]PLYMOUTH MARINE LABORATORY, PROSPECT PLACE, WEST HOE, PLYMOUTH PL1 3DH, UK, [2]FRS MARINE LABORATORY, VICTORIA ROAD, ABERDEEN AB11 9DB, UK, [3]DEPARTMENT OF ZOOLOGY, UNIVERSITY OF ABERDEEN, TILLYDRONE AVENUE, ABERDEEN AB9 2TN, UK AND [4]INSTITUT FÜR OSTSEEFORSCHUNG WARNEMÜNDE SEESTRAßE 15, D18119 ROSTOCK, GERMANY

*CORRESPONDING AUTHOR: pkw@pml.ac.uk

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Analysis of the demographic structure of Calanus species in the North Atlantic presents particular difficulties due to the overlapping spatial distributions of four main congeneric species (Calanus finmarchicus, Calanus helgolandicus, Calanus glacialis and Calanus hyperboreus). These species have similar morphologies, making microscopic discrimination only possible between some of the species at late copepodite or adult stages. However, molecular techniques now offer the possibility of screening significant numbers of specimens and unambiguously identifying them to species, regardless of developmental stage. Unfortunately, the processing rate of specimens by molecular methods is still too low to offer a realistic alternative to microscopy for analysis of samples from large field surveys. Here, we outline and test an approach involving the use of molecular methodology in conjunction with conventional microscopy to assess the species assignment of developmental stage abundances of Calanus congeners. Our study has highlighted many important methodological issues. First, it cannot be assumed that the species composition is homogeneous across the development stages; applying proportional species composition of adults to morphologically undistinguishable earlier development stages can result in error. The second important conclusion is that prosome length may be a highly unreliable discriminator of C. finmarchicus and C. glacialis.

INTRODUCTION

Fundamental to investigating marine zooplankton dynamics, community structure and diversity is the correct identification of species. Owing to the similar morphologies and restricted diagnostic features of zooplankton, positive identification is frequently complicated. Copepod communities are no exception, and correct identification of the different species within a genus presents a persistent problem due to the incidence of congeneric species.

Distinguishing the congeneric *Calanus* species in the North Atlantic has attracted particular attention, because the main subpolar species *Calanus finmarchicus* plays such an important role in the ecosystem as a grazer of phytoplankton and ciliates (Irigoien *et al.*, 1998; Meyer-Harms *et al.*, 1999), and as a major food source for larvae, juveniles and adults of commercially important fish (Runge, 1988; Runge and DeLafontaine, 1996; Gaard and Reinert, 2002; Gislason and Astthorsson, 2002; Ringuette *et al.*, 2002; Beaugrand *et al.*, 2003). In addition, *C. finmarchicus* abundance is known to vary with changing climatic conditions (Fromentin and Planque, 1996; Greene and Pershing, 2000; Beaugrand and Reid, 2003), and its gradual replacement by the morphologically similar subtropical congener *Calanus helgolandicus* at the southern edge of its latitudinal range in the eastern

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Atlantic is considered to be an indication of climate change. At the northern edge of its latitudinal range, *C. finmarchicus* overlaps with the polar congeners *Calanus hyperboreus* and *Calanus glacialis* (Barnard *et al.* 2004; Beaugrand, 2004).

Morphologically, *C. finmarchicus*, *C. helgolandicus* and *C. glacialis* are very similar, with diagnostic features essentially being restricted to size or minor variations in secondary sexual characteristics (Rees, 1949; Jaschnov, 1957; Matthews, 1967). Microscopic discrimination is possible only by experienced taxonomists at the late copepodite and adult stages; identification of early copepodite and nauplii stages by microscope is still virtually impossible. *Calanus finmarchicus* and *C. helgolandicus* can only be discriminated at copepodite stages C5 and C6 (Frost, 1974; Fleminger and Hulsemann, 1977), but for *C. glacialis* and *C. finmarchicus*, even C5 and C6 stages present a significant problem having only very minor differences in morphology (Frost, 1971; Fleminger and Hulseman, 1977). The usual approach to enumeration of these two species in mixed samples is to apportion the population on the basis of prosome length (Head *et al.*, 1999) since *C. glacialis* are generally larger, though the two species do have an overlapping size range (*C. finmarchicus* 1.4–2.2 mm; *C. glacialis* 2.0–2.8 mm; Hirche, 1991). *Calanus hyperboreus* copepodites are readily discriminated from the other congeners by size, but nauplii remain difficult to distinguish. Historically, the geographic location of collection has, at least partially, been relied upon as an indicator of species identity.

Molecular techniques have the potential to provide definitive species identification, thereby overcoming the taxonomic difficulties faced by microscopists. In the case of the North Atlantic *Calanus* congeners, the mitochondrial gene encoding the large subunit of ribosomal RNA (the 16S rRNA gene or 16S rDNA) shows considerable base sequence divergence between the species (Bucklin *et al.*, 1995). Utilizing both interspecific variation and intraspecific conservation of the 16S rRNA gene, a molecular method to provide definitive identification of individuals to species has been developed (Lindeque *et al.*, 1999, 2004). The system involves amplification of a region of the 16S rRNA gene using the polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis of the amplified product. Using this technique, it is possible to unambiguously identify *Calanus* to species at any developmental stage from egg to adult.

Unfortunately, molecular and microscopical methods for species identification of plankton have very different capabilities in terms of specimen throughput. A significant scale field survey needs to collect a large number of samples to resolve three-dimensional distributions of plankton. In turn, many specimens of each target species need to be enumerated per sample to achieve a statistically robust estimate of abundance. Hence, if the requirement is for species and stage-resolved demographic data, then the method chosen needs to be capable of enumerating a considerable number of specimens of each target species. Automated analysis of microscope images of mixed plankton can process large numbers of specimens per day including sample handling time, but species and stage discrimination is even less resolved than by a human analyst. Specimen throughput by a trained microscopy analyst is obviously less than by automated analysis, but the level of discrimination is higher. Specimen throughput by a semirobotic molecular method is considerably less than by microscopists; however, the technique does provide identification of the highest precision.

The main objective of the work described here was to develop a method for integrating molecular and microscopic analysis of plankton samples to increase the precision of species resolution in results from large field surveys. Microscopic analysis by trained analysts remains the main vehicle for estimating the abundance of taxonomic groups of zooplankton on account of the high throughput, but molecular methods are used to enhance the species resolution of the taxonomic groups enumerated by the microscopists. The need for this initiative arose from the key field studies of the main UK-GLOBEC Programme, Marine Productivity, which focussed on plankton production and especially *C. finmarchicus* population dynamics in the Irminger Sea. This is a mixing area for warm Atlantic water carried northwards by the Irminger Current and cold polar water travelling south with the East Greenland Current. Hence, both *C. finmarchicus* and *C. glacialis* could be expected in the survey region in varying proportions. In addition, the eastern part of the survey region encroaches on the oceanic distribution range of *C. helgolandicus*. Hence, the precision of *Calanus* species discrimination is an important issue in this region.

METHODS

Sample collection and storage

Sampling was carried out on four surveys on the RRS 'Discovery' (8 November–12 December 2001, 25 April–24 May 2002, 28 July–23 August 2002 and 15 November–13 December 2002). These surveys are referred to as D258, D262, D264 and D267, respectively, corresponding to the sequential cruise number of each survey. The spatial extent of each survey (Fig. 1a) varied principally according to weather conditions. In the main, they were centred on

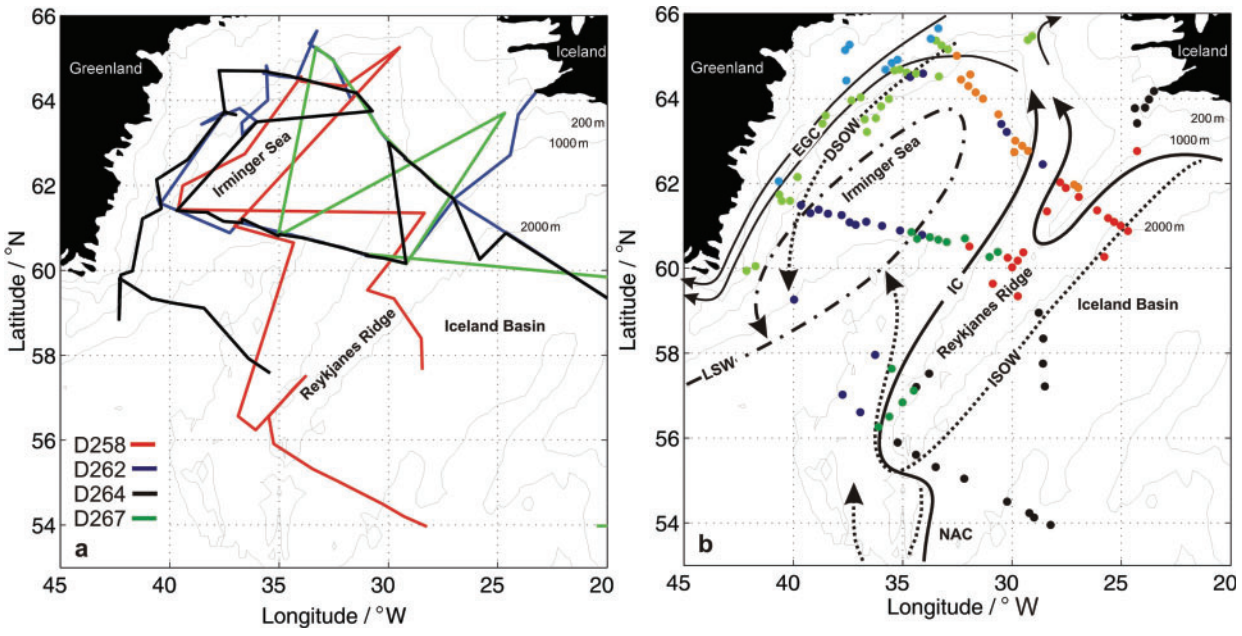

Fig. 1. (**a**) Cruise tracks of the four marine productivity cruises (D258, D262, D264 and D267) in the northern North Atlantic. (**b**) Circulation pathways of the Irminger Sea: Surface circulation is given by solid lines (NAC is North Atlantic Current, IC is Irminger Current and EGC is East Greenland Current). Mid-depth circulation is given by dot-dashed line (LSW is Labrador Sea Water). Deep circulation is given by dotted lines (ISOW is Iceland-Scotland Overflow Water, DSOW is Denmark Strait Overflow Water). Depth contours are 200, 1000, 2000, 3000 and 4000 m. Overlaid are all marine productivity stations. The colours represent the physical zones that the stations lie in (for details see Holliday *et al.*, 2006). The zones are: East Greenland Current, Atlantic origin (EGC-A, pale green), East Greenland Current, polar origin (EGC-P, pale blue), North Irminger Current (NIC, orange), South Irminger Current (SIC, dark green), Central Irminger Sea (CIS, dark blue), Reykjanes Ridge mode water (RRmw, red) and Iceland Basin water type (Ibwt, black).

the Irminger Sea basin, and the fringing East Greenland shelf, southwest Iceland shelf and the northern end of the mid-Atlantic Ridge, referred to as the Reykjanes Ridge. A few samples were collected further east in the Iceland Basin during each survey, but especially during D267 where many locations in the eastern Atlantic were sampled.

Plankton were sampled with either the ARIES system (Dunn *et al.*, 1993) or the OCEAN sampler (adapted from Dunn *et al.*, 1985; Sameoto *et al.*, 2000). Both samplers were designed to collect a sequential set of discrete plankton samples delineated by subsea pressure intervals during the descent and ascent legs of an oblique towed deployment. The ARIES used a 200-μm mesh net and stored a sequence of samples at intervals corresponding to 50 or 75 m depth intervals between surface, 3000 m depth and back to the surface. The OCEAN sampler carried an integrating net which was open throughout each tow (referred to as the pup-net), plus seven nets which were opened and closed in sequence on the ascent leg of each tow from 400 m to the surface, according to a pressure schedule corresponding to 100 m depth intervals between 400 and 100 m and 25 m intervals between 100 m and the surface. All the nets on the OCEAN sampler were of 95-μm aperture mesh.

The 200-μm mesh in the ARIES system retained *Calanus* spp. copepodite stages C1 and later, but not eggs or nauplii. The 95-μm fine mesh of the OCEAN sampler retained all development stages, but undersampled the late copepodites due to the smaller volume filtered per sample (typically 1–2 m^3 compared to 10–15 m^3 per ARIES sample).

The catches in the descent-leg nets from ARIES were preserved in 4% formalin and returned to the laboratory for microscopic analysis. The ascent-leg ARIES nets were examined by eye to determine which ones contained high numbers of *Calanus* (nets with many *Calanus* appeared reddish and oily). A few nets showing peak plankton concentrations were put on ice, and subsamples of *Calanus* spp. individuals for molecular analysis were sorted by developmental stage (C4, C5 and C6 male and female) under a dissecting microscope with cold light source. Animals were preserved in aliquots of 10 (where numbers allowed) in cryovials containing 95% ethanol, with a maximum of 10 per mL. The remainder of the bulk material from each ascent-leg sample was preserved in 4% formalin.

The depth-resolved fine mesh nets from OCEAN sampler were preserved in 4% formalin for microscopic analysis on return to the laboratory. The integrated 95-μm pup-net samples were stored in either 30 or 100 mL

of 95% ethanol, depending on the amount of material. These samples were also returned to the laboratory to be further sorted microscopically before molecular analysis.

Analysis of specimens from 200-μm mesh nets (ARIES)

Microscopic analysis

The formalin-preserved descent-leg ARIES nets were analysed at Aberdeen University through a series of multianalyst workshops to enumerate key taxonomic groups including *Calanus* spp. The numbers of individuals of each *Calanus* developmental stage C1–C6 were determined. Copepods were initially divided by size and then examined at a higher magnification to identify, where possible, the correct species and stage. *Calanus* spp. stages were identified by the number of urosome segments and the number of pairs of swimming legs. In addition, an elongated first basal urosome segment and a broadened base of the antennae were used to discriminate adult males and a swollen first urosome segment to identify adult females. *Calanus hyperboreus* were distinguished from other *Calanus* species by size. *Calanus finmarchicus* and *C. helgolandicus* stages C5 and C6 males and females were identified by the curvature of the inside of the fifth pair of swimming legs; earlier stages were indistinguishable as they do not show this difference in curvature. *Calanus glacialis* was not distinguishable from *C. finmarchicus* and *C. helgolandicus* at any stage by routine microscopy.

The same criteria were used for the microscopic analysis onboard ship to obtain the subsamples of *Calanus* species picked from the ascent leg of the ARIES tows for molecular analysis. The exception being that the fifth pair of swimming legs could not be removed while onboard ship to examine proportions of *C. finmarchicus* and *C. helgolandicus*. *Calanus hyperboreus* were deliberately not picked as they were thought to be identifiable by their size alone.

Molecular analysis

Individual *Calanus* were identified to species according to the RFLP signature of their mitochondrial 16S rDNA, following PCR amplification, restriction digestion and agarose gel electrophoresis, using the molecular identification technique described by Lindeque *et al.* (Lindeque *et al.*, 1999). The technique was modified and partially automated to increase the processing rate. The final technique is described below.

Individual animals were rehydrated in 35 μL of MilliQ water in a 96-well plate for 6–12 h at room temperature. Following rehydration, 5 μL of ×10 JumpStart Taq DNA polymerase buffer (Sigma-Aldrich, Poole, Dorset, UK) was added. Copepods were homogenized using a 21G needle inserted into a pellet pestle homogenizer (Anachem, Luton, Bedfordshire, UK) and incubated overnight covered at 4°C. After incubation, the remaining reaction components were added [5 μL 2 mM dNTPs, 2.5 μL each of primers 16SAR and 16SB2R (100 ng μL^{-1}), and 2 U JumpStart Taq DNA polymerase (Sigma-Aldrich)]. The amplification primers used were 16SAR (5′-CGCCTGTTTAACAAAAAC AT-3′; Palumbi and Benzie, 1991) and 16SB2R (5′-AT TCAACATCGAGGTCACAAAC-3′; custom designed from existing *Calanus* sequence data). Amplifications were carried out in either a standard thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, Warrington, Cheshire, UK) or the thermal cycler on a robotic workstation (RoboSeq 4204S, MWG Biotech, Milton Keynes, Buckinghamshire, UK). The cycling parameters included an initial denaturation step at 94°C (5 min) followed by 40 cycles of 45°C (2 min), 72°C (1 min) and 94°C (1 min). A final annealing phase at 45°C (2 min) was followed by an extension phase at 72°C (5 min) and storage at 4°C until use. Amplification efficiency was checked by analysing a random selection of 10 μL aliquots by agarose gel electrophoresis (1.5%).

Restriction digests were performed on a 15 μL aliquot of each amplification by the addition of 0.5 μL bovine serum albumin (1 mg mL^{-1}) and 2.5 U of each restriction enzyme *Dde* I and *Vsp* I. Incubations were performed at 37°C for 1 h. The digestion products were separated by electrophoresis through a 2% metaphor agarose gel, prechilled for 30 min at 4°C to improve resolution. The gels were observed and photographed by UV transillumination.

The majority of the manipulations were performed using a robotic molecular biology platform (RoboSeq 4204S, MWG Biotech).

Prosome length measurements

For a subset of the ARIES nets, the prosome lengths of individual specimens of *Calanus* spp., stages C4–C6, were measured under a compound microscope using a calibrated eyepiece graticule. At the magnification used, the resolution of the measurements was estimated to be approximately 50 microns.

Analysis of prosome length data

Initially, the individual measurements of prosome length were grouped by development stage (C4, C5, C6 female or C6 male), biozone, depth and cruise, following the methods described below for the aggregation of molecular analyses of specimens from the ARIES samples. For groups with more than 30 individuals measured, length frequency distributions were then derived with 50 μm length class intervals.

The length frequency distributions were analysed by iterative optimization (Nelder and Mead, 1965) to

estimate the characteristics (mean and standard deviation) and relative proportions of constituent normally distributed populations. The only prior assumption was the number of constituent populations (assumed to be either 1 or 2). In most cases, it was clear whether the observed population comprised one or two components. Where there was doubt, the data were analysed twice, first assuming one component, then assuming two, and the goodness-of-fit taken as the criterion for deciding the final number of components. Fitting was achieved by selecting initial values for the mean and standard deviation of lengths for each component, and their proportional contributions, synthesizing the implied length frequency distribution to the same class intervals as the observations and optimizing on the residual sum of squares between the derived and observed frequencies. The process was performed using the 'optim' function in the statistical package R (R Development Core Team, 2005).

Analysis of specimens from 95-μm mesh nets (OCEAN sampler)

N1–N3, N4–N6, C1, C2 and C3 *Calanus* were identified from the fine mesh samples from OCEAN sampler by microscopic analysis. The developmental stages of naupliі for all *Calanus* congeners were discriminated by the number and arrangement of the spines at the posterior end of the body and the number and arrangement of the setae on the distal segment of the first antenna. Copepodite stages were identified by the number of urosome segments and the number of pairs of swimming legs. These samples were used to enumerate the key taxonomic group of *Calanus* at each developmental stage N1–C3. There are no distinguishable morphological features suitable to discriminate between *C. finmarchicus*, *C. helgolandicus* and *C. glacialis* at any of these early developmental stages or between these congeners and *C. hyperboreus* at the nauplii stages.

Calanus early moult stages as described above were sorted from the ethanol-preserved integrated pup-net samples for molecular analysis. Groups of individuals at each moult stage were preserved in 1.5 mL vials containing 95% ethanol. Individual *Calanus* were identified to species using the molecular technique described above.

Integrating microscopic analysis and molecular analysis

It was only possible to perform molecular analyses on specimens from a small subset of the samples collected during the surveys, too few to permit haul-specific estimates of the *Calanus* speciation. Hence, it was necessary to aggregate the molecular data over several stations to provide statistically robust estimates of species composition for each developmental stage.

The principal requirement for any aggregation across stations was that the stations concerned must be drawn from a common zone hypothesized to be homogeneous with respect to *Calanus* speciation. As the basis for zonation, we used the zone definitions for the Irminger Sea developed by Holliday *et al.* (Holliday *et al.*, 2006). Briefly, these were distinct in terms of temperature, salinity, stratification and nutrient concentrations in the upper water column and persisted over an annual cycle and delineated the major water masses and current systems in the region. The assignment of sampling stations to zones together with the main circulation pattern is shown in Fig. 1b. Within each biozone, the data were also grouped according to depth as being shallower or deeper than 400 m. This depth horizon was chosen as separating the C4–C5 winter resting phase of *C. finmarchicus* from the spring and summer growth and reproductive phases (Heath *et al.*, 2004), and being the maximum deployment depth of the OCEAN sampler.

RESULTS

A total of 458 923 specimens of *Calanus* stages N1–C6 were analysed from the four surveys from formalin-preserved samples to give an estimate of abundance (D258: 25 734; D262: 159 717; D264: 257 422; D267: 16 050). Molecular analyses were successfully carried out on 5368 individuals of stage N1–C6 (D258: 468; D262: 1705; D264: 2890; D267: 305), and prosome lengths were measured on 27 890 individuals of stage C4–C6 (D258: 6842; D262: 9204; D264: 9934; D267: 1910).

Analysis of specimens from 200-μm mesh nets (ARIES)

Microscopic analyses

A total of 407 626 specimens from the formalin-preserved descent-leg ARIES nets were successfully analysed to give an abundance of *Calanus* at each developmental stage C1–C6.

Molecular analyses

The molecular technique allowed successful identification of *Calanus* to species for the stages C4, C5 and C6 removed from the ascent-leg of ARIES nets. Over 95% of copepods analysed were successfully amplified and digested to give a characteristic restriction profile for one of the four *Calanus* congeners during cruises D258, D262 and D267. The success rate of amplification of

specimens from D264 was slightly lower at just over 90%, most probably due to the first-time use of the robotic workstation during this cruise for analysing samples *in situ*. The molecular analyses of specimens from the Irminger Sea region showed that these were mostly *C. finmarchicus*, except in the East Greenland Current where *C. glacialis* was present at 2–20% of stage abundance in the upper 400 m (Table I). Below 400 m, the specimens analysed were all *C. finmarchicus*, except in the East Greenland Current–Polar zone, where one *C. glacialis* was identified from a depth of 450 m. *Calanus glacialis* were also present in the upper 400 m on the Reykjanes Ridge during D262.

During survey D267, the vessel visited seven stations in the eastern Atlantic (Rockall Trough and southern Iceland Basin). The molecular analyses of C4–C6 from this region indicated that approximately 80% were *C. finmarchicus*, the remainder being *C. helgolandicus* or *C. glacialis*.

Comparison of microscopic and molecular analyses—assessment of microscopist's success at discriminating C. helgolandicus

The microscopists who processed the formaldehyde-preserved samples from the ARIES nets were able to make an attempt at discriminating *C. helgolandicus* from *C. finmarchicus* by the curvature of the inside of the fifth pair of swimming legs at the late copepodite stages (C5 and C6). Very few *C. helgolandicus* were encountered in the samples from the Irminger Sea region, confirmed by their absence in the molecular analysis results. However, both the molecular analyses and the microscopists found a significant proportion of *C. helgolandicus* in the samples from the eastern Atlantic collected during D267. A station-by-station comparison of the estimated proportion of *C. helgolandicus* from these stations is shown in Fig. 2a and b. The data indicate that in most cases the microscopists overestimated the proportion of *C. helgolandicus*, although the number of specimens examined differed greatly between the two methods, and the confidence intervals for the molecular data are wider than those for microscopy.

Comparison between molecular and prosome length data from the Irminger Basin

The analysis of prosome length data gave a less distinct impression of the likely incidence of samples containing multiple congeneric species (Table II) compared to molecular analysis. Two-component length frequency distributions were found in the East Greenland Current, consistent with the identification of both *C. finmarchicus* and *C. glacialis* by molecular analysis, but also in the North Irminger Current and Reykjanes Ridge zones where the molecular data indicated that the population was almost exclusively composed of *C. finmarchicus*. On the other hand, length frequency distributions were exclusively unimodal in the central Irminger Sea, South Irminger Current and Iceland Shelf zones, where the molecular data indicated a monospecific population of *C. finmarchicus*.

Considering the instances of conjoint data from both molecular and prosome length analyses (Table III), out of 52 cases the molecular analyses identified nine of mixed *C. finmarchicus*/*C. glacialis*. The prosome length analyses identified 13 cases of two-component population, but, assuming that the molecular data were definitive, nine of these (69%) were false-positives (Table III). Conversely, the molecular analyses identified 43 of the 52 conjoint cases to be exclusively *C. finmarchicus*, whilst the prosome length measurements indicated 39 cases of unimodal distribution. Of these, five (13%) were in fact mixed according to the molecular analyses. Hence, the data indicate a high failure rate for prosome length data as a means of discriminating between *C. finmarchicus* and *C. glacialis* in this particular survey region.

Analysis of specimens from 95-μm mesh nets (OCEAN sampler)

Microscopic analysis

Significant numbers of naupliar and juvenile copepodite stages (C1–C3) of *Calanus* spp. were caught only during the spring and summer surveys (D262 and D264). Quantitative sampling for these stages with the 95-μm mesh OCEAN sampler was restricted to the upper 400 m, and no significant incidental catches in the coarser mesh ARIES samples were noted. A total of 51 297 *Calanus* early moult stages N1–C3 were identified from the fine mesh samples from OCEAN sampler by microscopic analysis to give an estimate of abundance.

Molecular analysis

Molecular identification was successfully performed on each developmental stage of *Calanus* (N1–C3) sorted from the OCEAN sampler 95-μm mesh nets. The success rate of amplification and subsequent digestion to produce a characteristic restriction profile was considerably lower (83%) than the molecular analyses of the copepods sorted from the ARIES samples. This was not attributed to the smaller sizes of the specimens being identified but could be linked to the storage conditions of the OCEAN sampler pup-nets prior to extraction of the individuals for molecular analysis. Where there was an overabundance of material in a single pup-net, the storage conditions, including the size of container and amount of ethanol used, did not appear sufficient to allow the ethanol to

Table I: Species composition as a proportion of total Calanus for stages C4-C6 analysed by molecular methods from the 200-μm mesh nets of the ARIES system from each depth layer, zone and cruise

Cruise/depth	C4			C5			C6f			C6m		
	fin	*hel*	*gla*	*fin*	*hel*	*gla*	*fin*	*hel*	*gla*	*fin*	*hel*	*gla*
Region: Central Irminger Sea												
D258-s				1	0	0						
D262-s							1	0	0			
D264-s	1	0	0	1	0	0						
D267-s												
D258-d				1	0	0						
D262-d							1	0	0			
D264-d	1	0	0	1	0	0	1	0	0			
D267-d	1	0	0	1	0	0						
Region: East Greenland Current–Atlantic												
D258-s				0.8	0	0.2						
D262-s	1	0	0	1	0	0	0.988	0	0.012	1	0	0
D264-s	0.867	0	0.133	0.972	0	0.028	1	0	0			
D267-s												
D258-d				1	0	0						
D262-d												
D264-d	1	0	0	1	0	0	1	0	0			
D267-d												
Region: East Greenland Current-Polar												
D258-s												
D262-s				0.778	0	0.222	1	0	0	0.8	0	0.2
D264-s	0.909	0	0.091	0.917	0	0.083	1	0	0			
D267-s												
D258-d				0.9	0	0.1						
D262-d												
D264-d												
D267-d												
Region: Northern Irminger Current												
D258-s												
D262-s	1	0	0	1	0	0	1	0	0			
D264-s	1	0	0	1	0	0						
D267-s				1	0	0						
D258-d				1	0	0						
D262-d												
D264-d	1	0	0	1	0	0						
D267-d				1	0	0						
Region: Reykjanes Ridge												
D258-s												
D262-s				0.975	0	0.025	1	0	0	0.857	0	0.143
D264-s	1	0	0	1	0	0	1	0	0			
D267-s				1	0	0						
D258-d												
D262-d												
D264-d	1	0	0	1	0	0						
D267-d				1	0	0						
Region: Southern Irminger Current												
D258-s												
D262-s							1	0	0			

(continued)

Table I: continued

Cruise/depth	C4			C5			C6f			C6m		
	fin	*hel*	*gla*	*fin*	*hel*	*gla*	*fin*	*hel*	*gla*	*fin*	*hel*	*gla*
D264-s	1	0	0	1	0	0	1	0	0	1	0	0
D267-s												
D258-d				1	0	0						
D262-d												
D264-d	1	0	0	1	0	0						
D267-d												
Region: Iceland Shelf												
D258-s												
D262-s	1	0	0	1	0	0						
D264-s												
D267-s	1	0	0	1	0	0						
D258-d												
D262-d												
D264-d												
D267-d												
Region: Outside basin												
D258-s												
D262-s	1	0	0	1	0	0	1	0	0			
D264-s												
D267-s				0.784	0.196	0.02	0.4	0	0.4			
D258-d	1	0	0	1	0	0						
D262-d												
D264-d	1	0	0	1	0	0						
D267-d												

fin, Calanus finmarchicus; *hel, Calanus helgolandicus*; *gla, Calanus glacialis*; s, shallow, above 400 m; d, deep, below 400 m.

penetrate the entire sample. Even considering specimens which were further extracted and stored in optimal conditions for molecular analysis, the DNA was too badly damaged to allow successful amplification.

The molecular analyses of nauplii and copepodite stages C1–C3 showed a surprisingly low incidence of *C. glacialis* (Table IV), especially for the D262 survey. Notable proportions of *C. glacialis* were found only among stage C3 in the East Greenland Current–Atlantic zone for D262. During D264, few *C. glacialis* were found at stages C1 and C2 in the East Greenland Current–Atlantic zone and in all stages C1–C3 in the East Greenland Current–Polar zone. This pattern is different from the later copepodite stages collected with ARIES, where *C. glacialis* was consistently present in the East Greenland Current zone. Thus, the data indicate that *Calanus* distribution in the Irminger Sea region is strongly stage dependent (Fig. 3). The small numbers of *C. glacialis* among the naupliar and juvenile stages presumably imply that reproduction of this species was not particularly successful in the region and that later stages were immigrants to the area carried in from the north by the East Greenland Current.

Incidence of *C. helgolandicus* gives further indication that distribution of these congeners is stage dependent in the Irminger Sea region. A small percentage (<2%) of *C. helgolandicus* were found at stages N1–N3, N4–N6 and C1 at a depth <400 m in the East Greenland Current during cruises D262 and D264 (Table IV). However, no later copepodite stages of *C. helgolandicus* were identified from samples collected with either OCEAN sampler or ARIES in the Irminger Sea region.

The main *Calanus* species to co-occur with *C. finmarchicus* naupliar stages in the northern part of the survey region (East Greenland Current, Central Irminger Sea and North Irminger Current) was *C. hyperboreus*. This

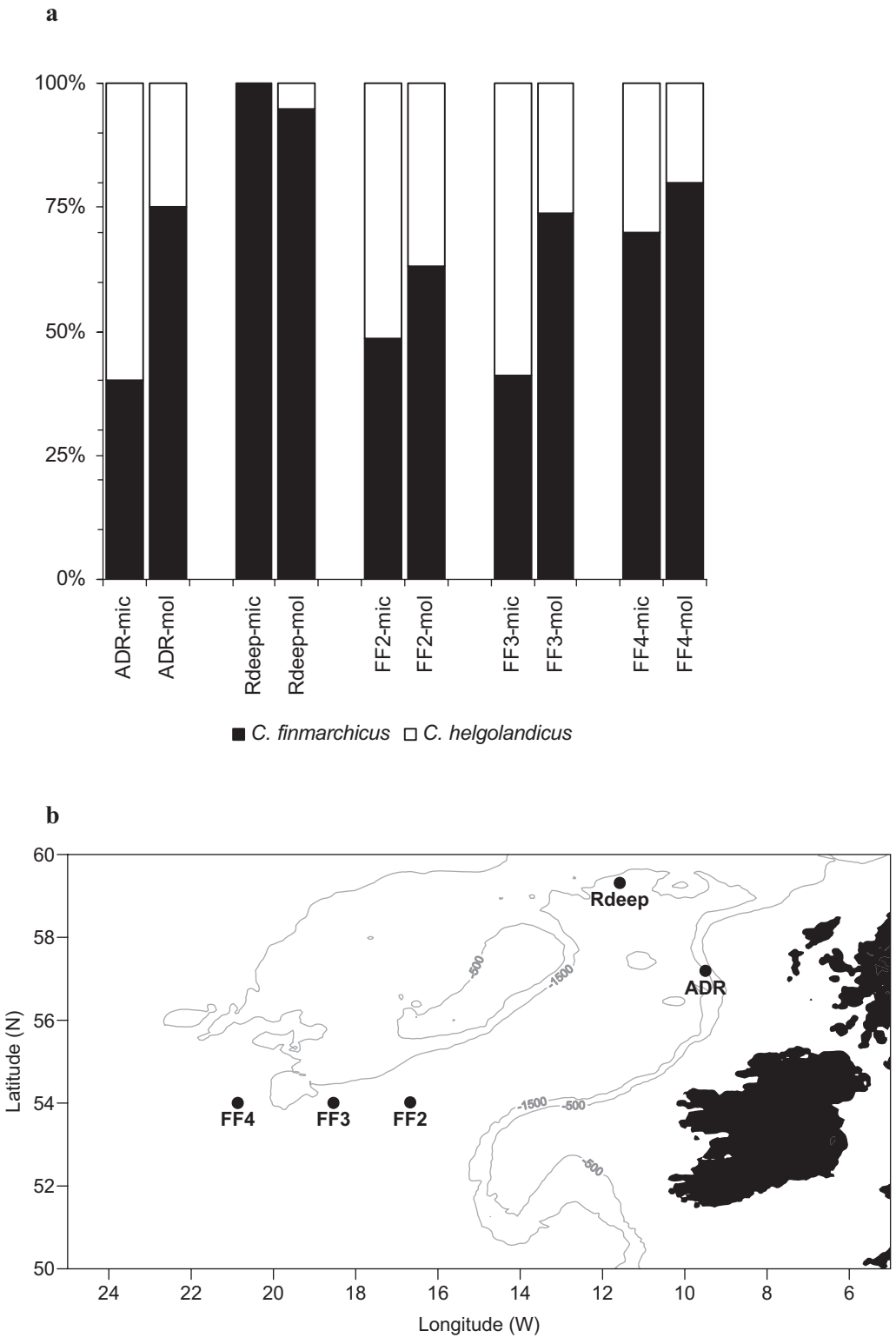

Fig. 2. (**a**) Comparison of molecular (mol) and microscopy (mic) discrimination of *Calanus finmarchicus* and *Calanus helgolandicus* (stage C5) collected with the 95-μm mesh nets of the ARIES system from five different stations in the eastern Atlantic. (**b**) Position of sampling stations [FF2, FF3, FF4, Rdeep (Rosemary deep) and ADR (close proximity to Anton Dohrn Seamount)] in the eastern Atlantic during cruise D267.

Table II: Statistics on the number of components making up the prosome length distribution of Calanus spp. from each depth layer, zone and cruise

Cruise/depth	C4			C5			C6f			C6m		
	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)
Region: Central Irminger Sea												
D258-s	1.60 (0.11)	0.92	1.98 (0.02)	2.12 (0.15)	1		–	–		–	–	
D262-s	1.72 (0.13)	1		2.20 (0.25)	1		2.87 (0.12)	1		2.46 (0.18)	1	
D264-s	1.67 (0.12)	1		2.25 (0.14)	1		2.68 (0.17)	1		2.59 (0.10)	1	
D267-s	–	–		–	–		–	–		–	–	
D258-d	1.72 (0.13)	1		2.28 (0.21)	1		2.97 (0.18)	1		–	–	
D262-d	–	–			–					–	–	
D264-d	1.75 (0.13)	1		2.28 (0.20)	–		2.71 (0.28)	1		–	–	
D267-d	1.69 (0.14)	1		2.19 (0.12)	0.74	2.51 (0.06)	2.71 (0.17)	1		–	–	
Region: East Greenland Current–Atlantic												
D258-s	–	–		2.12 (0.15)	1		–	–		–	–	
D262-s	1.72 (0.13)	1		1.98 (0.11)	0.24	2.32 (0.16)	1.92 (0.04)	0.02	2.64 (0.21)	2.45 (0.15)	1	
D264-s	1.67 (0.12)	1		2.05 (0.10)	0.40	2.28 (0.12)	2.33 (0.11)	0.41	2.70 (0.21)	2.53 (0.17)	1	
D267-s	–	–		–	–		–	–		–	–	
D258-d	1.67 (0.12)	1		2.24 (0.19)	1		2.60 (0.32)	1		–	–	
D262-d	–	–		–	–		–	–		–	–	
D264-d	1.73 (0.11)	1		2.21 (0.21)	1		2.74 (0.15)	1		–	–	
D267-d	–	–		–			–	–		–	–	
Region: East Greenland Current–Polar												
D258-s	–	–		2.12 (0.21)	0.83	2.87 (0.22)	–	–		–	–	
D262-s	1.68 (0.18)	–		2.07 (0.13)	–		2.63 (0.18)	0.73	2.77 (0.01)	–	–	
D264-s	1.69 (0.18)	–		2.17 (0.08)	0.17	2.26 (0.24)	2.31 (0.08)	0.32	2.59 (0.24)	2.50 (0.17)	–	
D267-s	–	–		–	–		–	–		–	–	
D258-d	1.81 (0.21)	0.67	2.09 (0.10)	2.32 (0.27)	0.92	2.52 (0.02)	2.64 (0.24)	–		–	–	
D262-d	–	–		–	–		–	–		–	–	
D264-d	–	–		–	–		2.59 (0.17)	–		–	–	

(continued)

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Table II: continued

Cruise/depth	C4			C5			C6f			C6m		
	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)
D267-d	–	–		–	–		–	–		–	–	
Region: Northern Irminger Current												
D258-s	–	–		2.17 (0.08)	0.62	2.48 (0.08)	–	–		–	–	
D262-s	1.79 (0.03)	0.29	1.68 (0.13)	2.25 (0.19)	1		2.62 (0.18)	0.86	2.77 (0.02)	2.39 (0.15)	1	
D264-s	1.58 (0.06	0.44	1.67 (0.17)	2.21 (0.12)	0.69	2.46 (0.07)	2.60 (0.23)	1		–	–	
D267-s	–	–		–	–		–	–		–	–	
D258-d	1.68 (0.11)	1		2.25 (0.16)	1		–	–		–	–	
D262-d	–	–		–	–		–	–		–	–	
D264-d	1.70 (0.09)	0.65	2.40 (0.27)	2.26 (0.20)	1		–	–		–	–	
D267-d	1.59 (0.12)	1		2.31 (0.19)	1		–	–		–	–	
Region: Reykjanes Ridge												
D258-s	–	–		–	–		–	–		–	–	
D262-s	1.72 (0.16)	1		2.21 (0.23)	1		2.41 (0.16)	1		–	–	
D264-s	1.67 (0.12)	1		2.17 (0.18)	1		2.49 (0.17)	0.85	2.83 (0.05)	2.58 (0.12)	1	
D267-s	–	–		–	–		–	–		–	–	
D258-d	–	–		2.25 (0.20)	1		–	–		–	–	
D262-d	–	–		–	–		–	–		–	–	
D264-d	1.69 (0.12)	1		2.28 (0.20)	1		–	–		–	–	
D267-d	1.59 (0.10)	0.92	1.99 (0.01)	2.33 (0.20)	1		–	–		–	–	
Region: Southern Irminger Current												
D258-s	–	–		–	–		–	–		–	–	
D262-s	1.61 (0.10)	1		2.05 (0.19)	1		2.45 (0.16)	1		2.19 (0.10)	1	
D264-s	1.66 (0.11)	1		2.23 (0.13)	1		2.54 (0.18)	1		–	–	
D267-s	–	–		–	–		–	–		–	–	
D258-d	1.59 (0.13)	1		2.24 (0.20)	1		–	–		–	–	
D262-d	–	–		–	–		–	–		–	–	
D264-d	1.68 (0.12)	1		2.32 (0.16)	1		–	–		–	–	
D267-d	–	–		–	–		–	–		–	–	
Region: Iceland Shelf												
D258-s	–	–		–	–		–	–		–	–	

(continued)

Table II: continued

Cruise/depth	C4			C5			C6f			C6m		
	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)	Component 1 Mean (SD)	Component 1 Proportion	Component 2 Mean (SD)
D262-s	1.60 (0.10)	1		2.05 (0.15)	1		2.35 (0.19)	1		2.14 (0.18)	1	
D264-s	–	–		–	–		–	–		–	–	
D267-s	1.66 (0.12)	1		2.11 (0.23)	1		–	–		–	–	
D258-d	–	–		–	–		–	–		–	–	
D262-d	–	–		–	–		–	–		–	–	
D264-d	–	–		–	–		–	–		–	–	
D267-d	1.74 (0.11)	1		2.27 (0.14)	0.80	1.88 (0.02)	–	–		–	–	
Region: Outside basin												
D258-s	–	–		–	–		–	–		–	–	
D262-s	–	–		–	–		–	–		–	–	
D264-s	–	–		–	–		–	–		–	–	
D267-s	–	–		–	–		–	–		–	–	
D258-d	–	–		–	–		–	–		–	–	
D262-d	–	–		–	–		–	–		–	–	
D264-d	–	–		–	–		–	–		–	–	
D267-d	1.59 (0.10)	0.92	1.99 (0.01)	2.18 (0.17)	1		–	–		–	–	

The mean length in millimetre and standard deviation is shown for each component, together with the estimated proportion of the population represented by component 1. Where only one component was detected, this proportion is 1. s, shallow, above 400 m; d, deep, below 400 m.

Table III: Frequency of mono- and duo-species populations in conjoint analyses of molecular and prosome length data of stage C4-C6 Calanus from the ARIES system

	Molecular analysis			
	One species present		Two species present	
	One-size component	Two-size components	One-size component	Two-size components
Prosome length analysis				
CIS	8	0	0	0
EGC-A	6	2	2	2
EGC-P	0	2	2	2
NIC	5	4	0	0
RR	6	1	1	0
SIC	7	0	0	0
IS	2	0	0	0
Total	34	9	5	4

CIS, Central Irminger Sea; EGC-A, East Greenland Current–Atlantic; EGC-P, East Greenland Current–Polar; NIC, Northern Irminger Current; RR, Reykjanes Ridge; SIC, Southern Irminger Current; IS, Icelandic Shelf.

Table IV: Species composition as a proportion of total Calanus for stages N1–C3 analysed by molecular methods from the 95-μm mesh nets of the OCEAN sampler system from each depth layer, zone and cruise

Cruise/depth	N1–N3				N4–N6				C1			C2			C3		
	fin	*hel*	*gla*	*hyp*	*fin*	*hel*	*gla*	*hyp*	*fin*	*hel*	*gla*	*fin*	*hel*	*gla*	*fin*	*hel*	*gla*
Region: Central Irminger Sea																	
D262-s	0.96	0	0	0.04	1	0	0	0	1	0	0	1	0	0	1	0	0
D264-s	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0
Region: East Greenland Current–Atlantic																	
D262-s	0.94	0	0.01	0.04	0.89	0.02	0	0.09	1	0	0	1	0	0	0.87	0	0.13
D264-s	1	0	0	0	0.99	0.01	0	0	0.96	0.01	0.03	0.99	0	0.01	1	0	0
Region: East Greenland Current–Polar																	
D262-s	0.67	0	0	0.33	1	0	0	0	1	0	0	1	0	0	–	–	–
D264-s	0.95	0.02	0.03	0	0.96	0	0.04	0	0.93	0	0.07	0.93	0	0.07	0.98	0	0.02
Region: Northern Irminger Current																	
D262-s	0.97	0	0	0.03	1	0	0	0	1	0	0	1	0	0	1	0	0
D264-s	1	0	0	0	1	0	0	0	0.97	0	0.03	1	0	0	1	0	0
Region: Reykjanes Ridge																	
D262-s	1	0	0	0	0.98	0	0.02	0	1	0	0	1	0	0	1	0	0
D264-s	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0
Region: Southern Irminger Current																	
D262-s	1	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0
D264-s	1	0	0	0	1	0	0	0	1	0	0	–	–	–	1	0	0
Region: Iceland Shelf																	
D262-s	0.94	0	0.06	0	1	0	0	0	1	0	0	1	0	0	1	0	0
D264-s	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Not in zone																	
D262-s	1	0	0	0	0.86	0.14	0	0	1	0	0	1	0	0	1	0	0
D264-s	0.80	0.20	0	0	0.88	0.12	0	0	1	0	0	1	0	0	1	0	0

S, shallow, above 400 m; *fin, Calanus finmarchicus*; *hel, Calanus helgolandicus*; *gla, Calanus glacialis*; *hyp, Calanus hyperboreus*.

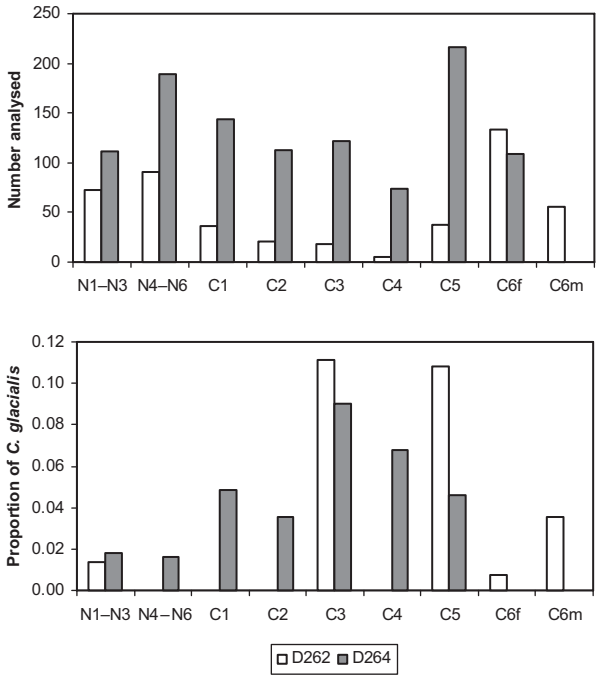

Fig. 3. Percentage of *Calanus glacialis* determined by molecular techniques for each development stage from nauplii through to C6 for the East Greenland Current zone for cruises D262 and D264.

species was not present in the molecular analysis results for C1–C6 since the copepodites are readily distinguishable by microscopy and were screened out of the specimens selected for preservation at sea.

Integration of molecular and microscopy data

The 0–400 m depth interval data from survey D262 was used as a case study for integrating the stage abundance estimates from microscopy with the percentage species compositions determined by molecular analysis. It was assumed that the proportional species composition was homogeneous for each stage within each zone. To test this hypothesis, a single-factor ANOVA test was used to compare species proportions for individual stations for the two Greenland Current zones, where the majority of *C. glacialis* were found (Table V). The test was performed for the zones and stages where there were results for more than two stations. The results indicated no significant difference in species composition between stations in each zone, suggesting that the individual stations were drawn essentially at random from a homogeneous distribution. The zonal compositions derived from the molecular analysis were applied to the microscopically derived stage abundance of every sample falling within each zone. Figure 4 shows semisynoptic maps of the stage abundance data apportioned into *C. finmarchicus* and *C. glacialis*.

DISCUSSION

Our study has highlighted many important methodological issues concerning the abundance and demography of congeneric *Calanus* species. First, it cannot be assumed that species composition is homogeneous across the development stages. In our case, the naupliar and early copepodite stages of *Calanus* spp. in the East Greenland Current were deficient in *C. glacialis* compared to the later copepodite stages, presumably due to species differences in reproductive success. Hence, although *C. finmarchicus* and *C. helgolandicus* can be reliably discriminated microscopically as C5 and C6 copepodites (as confirmed by our study), the practice of applying the proportional species composition of these stages to earlier development stages, which cannot be discriminated other than by molecular analysis (Heath *et al.*, 2000), is to be discouraged.

The second important conclusion is that prosome length may be a highly unreliable discriminator of *C. finmarchicus* and *C. glacialis*. The prosome length frequency distribution for a mixed *C. finmarchicus*/*C. glacialis* stage C4 population from the Greenland Sea presented by Hirche (Hirche, 1991; Fig. 2 therein) was

Table V: P-values for ANOVA test, comparing species-proportions from individual stations for the two Greenland Current zones (where the majority of C. glacialis was found)

Cruise	EGC-P				EGC-A			
	C4	C5	C6f	C6m	C4	C5	C6f	C6m
D262	–	0.5776	0.8177	0.9771	–	–	0.8744	–
D264	0.5993	0.7865	0.9375	–	0.6843	0.8118	–	–

Analysis was performed for the zones and stages where there were results for more than two stations to determine whether there was any significant difference in species composition between stations in each zone.

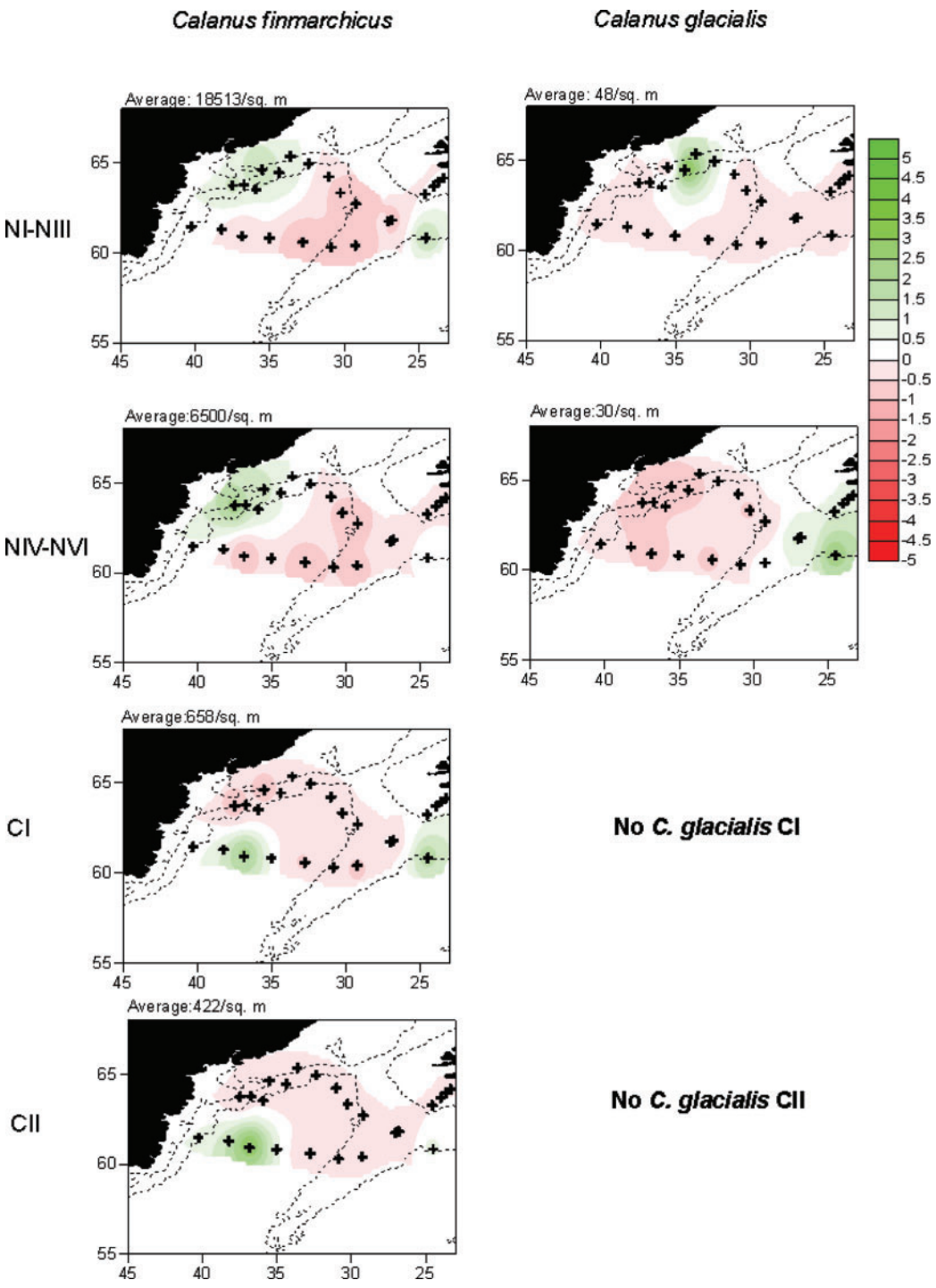

Fig. 4. Semisynoptic maps of *Calanus finmarchicus* and *C. glacialis* stage abundances for 0–400 m layer from D262 survey, showing the standard deviation at each station from the mean across the basin.

subjected to the component analysis programme developed for our project. The programme resolved two components—*C. finmarchicus*: mean prosome length 1.90 mm, SD 0.14 mm, proportion of the population 0.67; *C. glacialis*: mean prosome length 2.31 mm, SD 0.07 mm. Unfortunately, there were no conjoint

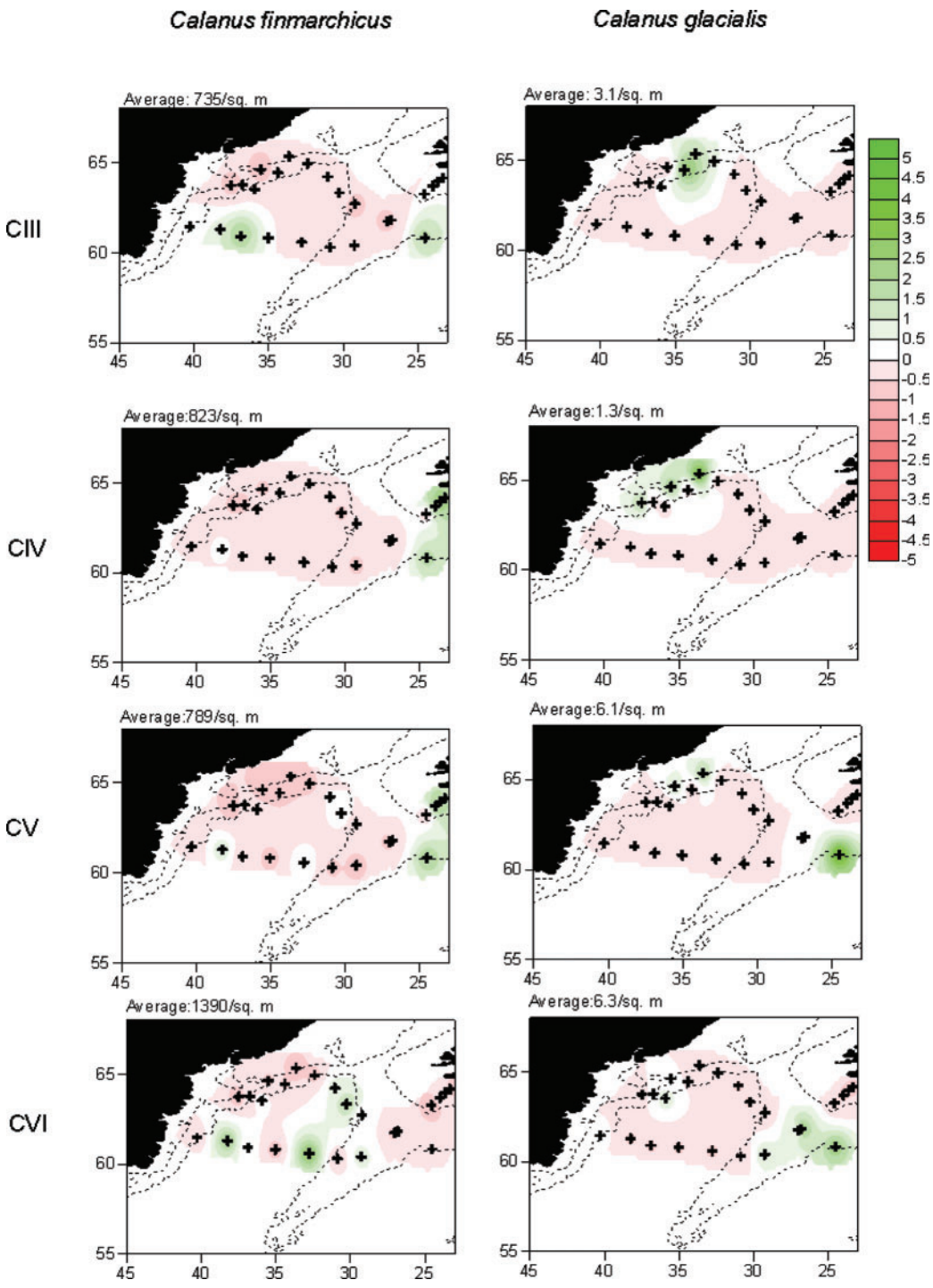

Fig. 4. continued

observations of mixed stage C4 *C. finmarchicus*/*C. glacialis* (by molecular analysis) and bimodal length distribution from our survey data for comparison. However, length data (with no accompanying molecular analysis) from D258, deeper than 400 m in the East Greenland Current–Polar zone, where other molecular data indicate the consistent incidence of *C. glacialis*, were bimodal. The data indicated a mean prosome length 1.80 mm,

SD 0.21 mm and proportion of the population 0.67 for *C. finmarchicus* and a mean prosome length 2.09 mm, SD 0.10 mm for *C. glacialis*. Hence, these data from the Irminger Sea suggest that *C. glacialis* is substantially smaller than reported by Hirche (Hirche, 1991) from the Greenland Sea. Hirche (Hirche, 1991) took all C4 >2.2 mm to be *C. glacialis*, and for his data from station 616, this criterion produced an estimate of 64% of the population being *C. finmarchicus*, compared to the 67% estimated by our component-splitting programme. However, the same criterion applied to our data for C4 in the East Greenland Current yields 92% *C. finmarchicus* compared to 67% estimated by our programme. Hirche (Hirche, 1991) cites prosome length criteria for separating later stages of *C. finmarchicus* and *C. glacialis* from the Greenland Sea and Barents Sea to be 3.0–3.1 mm for stage C5 and 3.2–3.32 mm for C6 females. These are all very much larger than even the mean lengths of the *C. glacialis* component for conjoint cases of mixed species by molecular analysis and bimodal size distribution in our data (*C. glacialis* component mean lengths: C5, 2.3 mm; C6 female, 2.6–2.8 mm).

Whilst the size distinction between the two species may be sufficiently large for effective separation in populations at high latitudes (e.g. in the Greenland Sea and Barents Sea), this is not the case at the southern fringe of the distribution of *C. glacialis*. In the Irminger Sea, the detection of bimodality in the length distribution gave a high incidence of false diagnoses of the presence or absence of *C. glacialis*, and the size criterion for assuming a specimen to be *C. glacialis* was clearly very different from that applicable at higher latitudes. The origin of the high percentage of false-positive diagnoses (i.e. bimodal length frequency distribution in a monospecific *C. finmarchicus* population) is unclear. One possibility is that stage C5 *C. finmarchicus* show a bimodal distribution of prosome length depending on their sex (Grigg *et al.*, 1981, 1987). There was also spatial variability in the mean length of *C. finmarchicus* across the region, with smaller-sized individuals in the warmer biozones (e.g. South Irminger Current) than in the colder zones (e.g. Iceland Shelf and East Greenland Current). Possibly, mixing of *C. finmarchicus* from different regions, especially in the North Irminger Current, could have given rise to bimodal length distributions.

Hence, we conclude that there is considerable variation in the size of *C. glacialis* over its distribution range, and in the Irminger Sea at the southern fringe of its range, its size distribution is too close to that of *C. finmarchicus* for prosome length to be used as a viable means of discrimination.

The outcome of our integration of microscopic and molecular data to separate abundances of different *Calanus* spp. at each moult stage in the Irminger Sea changes our perception of the spatial distributions of the *Calanus* congeners. Our interpretation of the distribution of *C. finmarchicus* when *C. glacialis* is taken into account is quite different from that which we would have obtained had we assumed that all *Calanus* spp. were *C. finmarchicus*. However, the additional analytical burden is large. It is clear that microscopy must remain the primary vehicle for estimating abundance for the foreseeable future. This remains the only practicable technique for enumerating the large number of specimens needed to establish credible demographic data. But, we have shown that molecular techniques have progressed beyond use as a strategic tool, towards application as a mass screening method, capable of dealing with respectable numbers of specimens. Furthermore, the method is equally applicable to the smallest naupliar stages as to the larger, more easily handled copepodites and adults, provided specimens are optimally stored.

In future programmes where discrimination of morphologically similar congeneric species is an analytical issue, we would strongly recommend the integration of molecular and microscopic techniques. On the basis of our experience, we would suggest consideration of zonal stratification of the samples collected at the outset, with the express aim of spreading the valuable molecular analysis capacity more evenly across the zones than we were able to achieve with our station-based subsampling scheme which left some zones under-sampled compared to others.

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